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Basic Study

Knockdown of Receptor for activated C kinase 1 by Regulating protein kinase C- ε reactive oxygen species Effectively Slows the Progression of Early Diabetic Retinopathy

RACK1/PKC-ε in early DR

Jian Tan, Ang Xiao, Lin Yang, Yulin Tao, Yi Shao, Qiong Zhou

Abstract

BACKGROUND

Diabetic retinopathy (DR) is a major ocular complication of diabetes mellitus, leading to visual impairment. Retinal pigment epithelium (RPE) injury is a key component of the outer blood retinal barrier, and its damage is an important indicator of DR. RACK1 activates protein kinase C-ε (PKC-ε) to promote the generation of reactive oxygen species (ROS) in RPE cells, leading to apoptosis. Therefore, we hypothesize that the activation of RACK1 under hypoxic/high-glucose conditions may promote RPE cell apoptosis by modulating PKC-ε/ROS, thereby disrupting the barrier effect of the outer blood retinal barrier and contributing to the progression of diabetic retinopathy.

AIM

To investigate the role and associated underlying mechanisms of RACK1 in the development of early diabetic retinopathy (DR).

METHODS

In this study, Sprague-Dawley rats and ARPE-19 cells were used as *in vivo* and *in vitro* models, respectively, to explore the role of RACK1 in mediating PKC-ε in early DR. Furthermore, the effect on the apoptosis and barrier function of retinal pigment epithelium (RPE) cells was also investigated in the former model.

RESULTS

Streptozotocin (STZ)-induced diabetic rats showed increased apoptosis and up-regulated expression of RACK1 and PKC-ε proteins in RPE cells following a prolonged modeling time. Similarly, ARPE-19 cells exposed to high glucose and hypoxia displayed elevated mRNA and protein levels of RACK1 and PKC-ε, accompanied by increases in reactive oxygen species production, apoptosis rate, and monolayer permeability. However, silencing RACK1 significantly downregulated the expression of PKC-ε and ROS, reduced cell apoptosis and permeability, and protected barrier function.

CONCLUSION

RACK1 plays a significant role in the development of early diabetic retinopathy, and may serve as a potential therapeutic target for DR by regulating the apoptosis and barrier function of RPE cells.

Key Words: Diabetic retinopathy; RACK 1; PKC- ε; ARPE-19

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Core Tip: To investigate the role and associated underlying mechanisms of RACK1 in the development of early diabetic retinopathy (DR). In this study, Sprague-Dawley rats and ARPE-19 cells were used as *in vivo* and *in vitro* models, respectively, to explore the role of RACK1 in mediating PKC-ε in early DR. RACK1 plays a significant role in the

development of early diabetic retinopathy, and may serve as a potential therapeutic target for DR by regulating the apoptosis and barrier function of RPE cells.

INTRODUCTION

Diabetic retinopathy (DR) is the main ocular complication of diabetes mellitus(DM), and a common cause of visual impairment and blindness. The International Diabetes Federation (IDF) predicts that the number of DM patients worldwide will increase from 460 million in 2019 to 700 million in 2045 [1], and approximately 30% of DM patients will eventually develop DR [2]. If effective treatment is not provided, DR patients will soon experience visual impairment or even blindness, so precise treatment in the early stage of DR is particularly important[3]. Retinal pigment epithelium (RPE) is the main component of the outer blood retinal barrier (oBRB), and leakage caused by BRB injury is a sign of DR[4]. However, to date, research on DR has mostly focused on the internal retinal barrier, while research on the damage mechanism of the oBRB in diabetes is limited.

In the early stage, through bioinformatics research on retinal tissue samples of DR patients and normal people without diabetes, our research team obtained four hub genes in total, and found that only the receptor for activated protein kinase C1 (RACK1) was the most highly expressed and the most differentially expressed (P = 0.003) hub gene (P = 0.003)[5]. RACK1 is a multifunctional signal transduction protein, also known as the anchoring protein of protein kinase C (PKC) [6]. PKC is a serine/threonine kinase involved in signal transduction, that can respond to the stimulation of specific hormones, neurons and growth factors [7]. The PKC family consists of 12 subtypes, among which PKC-α, -β, -δ and-ε are activated and play an important role in the occurrence and development of DR [7,8]. Among them, PKC-ε can enhance the activity of NADPH oxidase, thus promoting the production of reactive oxygen species (ROS) in RPE cells. The overaccumulation of ROS induces mitochondrial damage, apoptosis, inflammation, lipid peroxidation, and structural and functional changes in the retina [9].

Based on the above research, we hypothesized that the activation of RACK1 under hypoxic/high-glucose conditions might promote the apoptosis and migration of RPE cells by modulating PKC-ε/ROS, thereby disrupting the barrier effect of oBRB and leading to the progression of early DR. Therefore, this study was conducted to explore the therapeutic effect of PKC reduction induced by knockdown of RACK1-ε/ROS damage to oBRB on inhibiting early DR progression. Retinal pigment epithelium cells are highly polarized monolayer cells, usually induced by high glucose to simulate the DR environment[10]. Considering that high glucose (HG) concentration and hypoxia are the two main components in the environment of diabetes, we used both hypoxia and HG concentration to simulate the environment of diabetes [9].

MATERIALS AND METHODS

Animal model

All male Sprague-Dawley rats (8 wk old, weighing 180-220 g) were purchased from the Animal Center of Nanchang University. All rats were housed in standard rat cages under standardized environmental conditions, with controlled temperature (23 ± 2°C), humidity (50%), and a 12-h light/dark cycle. The diabetic group of rats were intraperitoneally injected with streptozotocin (STZ) (60 mg/kg body weight, Sigma-Aldrich; Merck Millipore , Darmstadt, Germany), dissolved in citrate buffer (pH 4.5), while the control group of rats received an equivalent volume of citrate buffer. Rats were considered diabetic if their blood glucose levels exceeded 16.7 mmol/L 72 h after STZ injection, and remained elevated for 1 wk. A total of 24 rats were included in the study, with 12 rats in each group. The rats were raised for 8 or 10 wk (n = 6 per group, respectively). All experiments were conducted in accordance with the guidelines for the care and use of laboratory animals and approved by the Medical Ethics Committee of the First Affiliated Hospital of Nanchang University.

Hematoxylin Eosin staining of the retina

Six rats from each group were sacrificed, and their eyeballs were harvested at 8 and 10 wk after successful modeling. The eyeballs were then fixed in a 20% paraformaldehyde

solution at 4℃ for 2 h. Subsequently, the samples were sectioned into 5 μm slices, stained with hematoxylin and eosin , and examined under a light microscope (magnification, 400×; Zeiss, OberCoring, Germany), to determine the number of RPE cells in the samples.

Cells and culture

Adult retinal pigment epithelial cell line-19 (ARPE-19) cells were purchased from Procell (Wuhan, China), and cultured in Dulbecco's modified Eagle's medium (DMEM) (containing 5.5 mM glucose) (Procell, Wuhan, China) supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA) and 1% penicillin/streptomycin (Thermo Fisher) in 5% CO2 at 37 °C. The cell high glucose and hypoxia model was chemically induced by adjustment of the glucose concentration of the culture medium to 25 mmol/L, while 400 μM cobalt chloride (CoCl2) (Merck, Germany) was added to the cell culture medium for 24 h before experimentation.

Small interfering RNA (siRNA) transfection

The RACK1-specific siRNA used in this study was obtained from Ruibo RIBOBIO (China), with the sequence 5′-3′ GTCTCTGGATCTCGAGATA. Transfection was performed in ARPE-19 cells when they reached 50%-70% confluency. The transfection was carried out using Lipofectamine 2000 (Invitrogen) with 100 nmol of RACK1 siRNA, and the medium was replaced with fresh medium 4-6 h after transfection. Transfected ARPE-19 cells were subsequently cultured for 48 h for mRNA experiments or 72 h for cell function and protein expression experiments.

Flow cytometry

ARPE-19 cells were grown in DMEM supplemented with 10% FBS for 12 h in a six-well plate, followed by treatment with high glucose and hypoxia for 24 h. The treated cells were collected (1 × 105 cells/mL) after digestion with pancreatic enzyme (Solarbio, Beijing, China) without EDTA, and then washed twice with pre-cooled PBS. The cells were resuspended in 100 μL of binding buffer, and stained with 5 μL Annexin V-FITC and 10 μL Propidium Iodide (Yeasen, Shanghai, China) for 15 min protected from light for 15 min. Subsequently, 400 μL of binding buffer was added to resuspend the cells.

The percentage of apoptotic cells was analyzed by flow cytometry (BD, FACSCalibur, USA).

Real-time quantitative polymerase chain reaction (RT– qPCR)

Total RNA was extracted at room temperature using TRIzol reagent (Invitrogen; Thermo Fisher Scientific) at room temperature, and immediately reverse transcribed immediately or stored at -80℃, as needed. mRNA was reverse transcribed into cDNA using the Strand cDNA Synthesis SuperMix for qPCR kit (11141ES60, Yeasen, China), and subsequently quantified using specific primers from the SANGON primer group (Shanghai, China) for each mRNA. RT-qPCR was performed using the ABI PRISM 145 real-time PCR system (Applied Biosystems; Thermo Fisher Scientific) using the Quick Start General SYBR Green (Roche, Basel, Switzerland). The cycle threshold (Ct) values were obtained and the relative mRNA expression levels were calculated based on the 2-ΔΔCt method. The oligonucleotide sequences of RT-qPCR primers are listed in Table 1.

Western blotting analysis

Crushed tissue or cells were lysed using a buffer (Thermo Fisher Scientific, Waltham, USA) to extract proteins. The protein extracts were subsequently separated using 10% SDS-PAGE, and the separated proteins were transferred onto a PVDF membrane (Amersham, Cytiva, Germany). To ensure proper sealing of the membrane, it was incubated with 10% skim milk powder for 2 h. To detect the target protein, the membrane was then incubated overnight at 4℃ with a primary antibody for GAPDH (1:1000, Abcam), along with RACK1 and PKC-ε. The following day, a secondary antibody (1:5000, Abcam) conjugated with horseradish peroxidase was incubated with the membrane at room temperature for 1.5 h. Finally, the membrane was treated with an ECL reagent (Amersham Pharmacia Biotech, Inc., USA), and the protein bands were visualized using ImageJ software (GE).

Determination of intracellular reactive oxygen species

ROS were measured by assessing the intracellular peroxide-dependent oxidation of DCFH-DA to produce the fluorescent compound 2′,7′-dichlorofluorescein (DCF). Cells were seeded in 24-well plates at a density of 2x104 cells per well, and cultured for 24 h.

After two washes with PBS, the cells were treated with fresh medium containing 25 mmol/L glucose and 400 μM cobalt chloride (CoCl2), and incubated for an additional 24 h. Subsequently, 20 μM DCFH-DA was added, and the cells were incubated for 30 min at 37°C. After two more washes with PBS, 400 μl of PBS was added to each well, and the fluorescence intensity was measured using a TECAN SPARK 450M (Tecan Group, Ltd., Manedoff, Switzerland).

Monolayer permeability assay

A 0.4 μm pore polycarbonate membrane insert with a 6.5 mm Transwell assay format (3413, Corning, NY, USA) was used to assess the vascular permeability of ARPE-19 cells. In each well, 105 cells in 200 μL of complete medium were transferred to the top chamber, while the bottom chamber was filled with 500 μL of the same medium. To conduct the permeability assays, the top chamber was loaded with 100 μL of a 1 mg/mL solution of fluorescein isothiocyanate dextran (FITC-dextran) (40 kDa, FD40, Sigma-Aldrich, St. Louis, MO, USA), while the bottom chamber was filled with 500 μL of phosphate-buffered saline. Following a 30-min incubation in the darkroom, 100 μL samples were collected from the bottom chamber and plated onto 96-well plates. The leakage of FITC-dextran was subsequently analyzed using a TECAN SPARK 450M (Tecan Group, Ltd., Manedoff, Switzerland), with an excitation wavelength of 490 nm, and an emission wavelength of 520 nm.

Statistical analysis

A minimum of three repetitions were conducted for each experiment. All data were presented as the means ± standard deviations. Statistical analyses were performed using Prism 9.0 software (GraphPad Software, San Diego, CA, USA), with Student's t-test and one-way ANOVA. A significance level of $P < 0.05$ was considered statistically significant.

RESULTS

Rats induced by STZ exhibited a typical diabetic phenotype

In our study, an animal model of diabetes was established through the injection of streptozotocin (STZ). To assess the progression of diabetes, both rat body weight and blood glucose levels were measured at weeks 0, 2, 4, 6, 8, and 10 (Figure 1). The results showed a significant increase in blood glucose levels in diabetic rats, which remained more than four times higher than those in the normal control group throughout the study period. Additionally, diabetic rats experienced significant weight loss compared to the normal control group.

In this study, we examined the number of cells in the RPE layer in each group at various time points following STZ injection. Cell counting was performed at 0, 2, 4, 6, 8, and 10 wk post-injection (Figure 2). There was no significant difference in the number of RPE cells between the control group (Sprague-Dawley rats) and the diabetic group at 0, 2, 4, and 6 wk after STZ injection ($P > 0.01$). However, at weeks 8 and 10, a notable decrease in the number of RPE cells was observed in the diabetic group. Furthermore, a significant decrease in the number of RPE cells was observed in the diabetic group compared to weeks 8 and 10 ($P < 0.01$). These findings suggested a progressive loss of RPE cells in the diabetic group over time.

Aberrant expression of RACK 1 and PKC- ε in the retinas of diabetic rats

The protein levels of RACK1 and PKC-ε in retinal tissues of normal and diabetic SD rats were assessed using western blot analysis at weeks 8 and 10 following STZ injection, as shown in Figure 3. The findings revealed that the protein levels of RACK1 and PKC-ε were significantly elevated in the retinal tissues of diabetic SD rats as compared to the normal group ($P <0.05$).

High glucose combined with hypoxia upregulated the transcription and protein levels of RACK 1 and PKC- ε in ARPE-19 cells

To mimic diabetes in an *in vitro* setting, ARPE-19 cells were subjected to high-glucose hypoxic conditions. The results, as illustrated in Figure 4, indicated that the transcription and protein levels of RACK1 and PKC-ε were significantly elevated in the high-glucose hypoxia group (Hg + Hypo) compared to the control group ($P <0.05$).

Silencing of RACK 1 in ARPE-19 cells under high-glucose hypoxia downregulated PKC- ε

We examined the mRNA and protein levels of PKC- ε by siRNA after siRNA-silencing of RACK 1 expression in ARPE-19 cells exposed to high glucose hypoxic conditions (Figure 5). The results showed that the inhibition of RACK 1 expression in ARPE-19 cells reduced PKC-ε transcription and protein levels under high glucose hypoxia ($P < 0.05$).

Silencing RACK 1 inhibits ROS elevation, apoptosis, and cell leakage in ARPE-19 cell monolayers under high-glucose hypoxia

We subsequently examined ROS levels, apoptosis, and permeability between monolayers after silencing RACK 1 expression by siRNA in ARPE-19 cells under high glucose hypoxia (Figure 6). The results showed that the inhibition of RACK 1 expression in ARPE-19 cells downregulated ROS levels, apoptosis, and cell permeability in monolayers under high glucose hypoxia ($P < 0.05$).

DISCUSSION

DR is a major ocular complication of diabetes that significantly impacts global health [11]. The mechanisms underlying its occurrence and development are complex and poorly understood [12]. Further elucidation of these mechanisms may aid in mitigating the progression of DR. RPE cells treated with high glucose are commonly used as an ideal model for investigating DR [13], and RPE cells are often exposed to high glucose combined with hypoxic conditions during DR development [14]. Studies have suggested that the disruption of RPE barrier function in DR may be due to the apoptosis of RPE cells under high glucose and hypoxic conditions [15], although the specific mechanisms remain unclear. In this study, we aimed to explore the effect of RACK1 on RPE barrier function through both *in vitro* and *in vivo* experimental models to verify its role in the occurrence and development of early DR.

The RPE consists of a highly specialized single-layer chromatophore, which is located between the microvessel of the villus and the outer segment of the photoreceptor [16,17].

The RPE and photoreceptors in the outer layer of the retina usually act as units to maintain normal visual function. This disorder, which is closely related to other ocular aberrations such as retinal detachment, will eventually lead to retinal degeneration and blindness. Similarly, mutations in photoreceptor cells or RPEs can lead to retinal degeneration [18]. In this study, we observed that the apoptosis of RPE cells began to occur in the retinas of STZ-induced diabetic rats at week 8. This suggests that apoptosis may be an important factor in the damage to the retinal RPE layer and the oBRB in early DR. Furthermore, we investigated the expression of RACK1 and PKC-ε in the retinal tissue of diabetic rats at weeks 8 and 10 using qPCR and western blot. Our results showed that both the mRNA and protein levels of RACK1 and PKC-ε were significantly higher in the retinal tissue of diabetic rats compared to the control group. Prior studies have similarly demonstrated that elevated levels of RACK1 can promote cell apoptosis induced by polyglutamine [19], while the inhibition of PKC-ε can protect RPE cells from lipopolysaccharide-induced injury. However, whether PKC-ε can be suppressed by regulating RACK1 remains unclear [20]. In contrast, inhibiting RACK1 may have the potential to reduce damage and cell apoptosis in RPE cells under diabetic conditions. Further investigations are needed to fully understand the mechanisms by which RACK1 and PKC-ε contribute to RPE cell damage and apoptosis in diabetes.

Therefore, in this study, we conducted an *in vitro* experiment to simulate the high glucose hypoxic environment of ARPE-19 cells in diabetes. We observed that the mRNA and protein levels of RACK1 and PKC-ε in ARPE-19 cells were significantly higher under high glucose hypoxic conditions compared to the control group. RACK1 serves as a scaffold protein which is recently found to mediated PKC activation[21]. PKC is a family of serine/threonine protein kinases that are crucial in regulating many biological processes, such as cell division, growth, and apoptosis, as well as cellular responses to environmental stressors. Meanwhile, the PKC pathway is also one of the important pathways involved in the mechanism of diabetic retinopathy.

However, the mRNA and protein levels of PKC-ε were significantly reduced in the control group after inhibition of RACK1 expression, suggesting that it is possible to

downregulate PKC-ε by inhibiting RACK1 expression. Hyperglycemia and tissue hypoxia in diabetes patients both increase the production of ROS, leading to retinal and tissue damage [22]. Our findings showed that the production of ROS by ARPE-19 cells was significantly increased under hyperglycemic and hypoxic conditions, but could be significantly reduced by inhibiting RACK1. PKC is known to be involved in ROS production, and the increase in PKC stimulates ROS production in the mammalian target of rapamycin complex 1 (mTORC1) pathway, which is related to autophagy [23]. PKC-ε play a tissue-specific role in redox biology, with specific isoforms being both a target of ROS and an up-stream regulator of ROS production[24]. Therefore, this effect may result in the accumulation of unfolded proteins and dysfunctional organelles in cells, contributing to the pathophysiology of diabetic retinopathy [25]. Furthermore, our study also revealed that under high glucose and hypoxic conditions, ARPE-19 cell viability decreased, and apoptosis increased. This could potentially be attributed to the activation of PKC-ε under high glucose and hypoxic conditions, leading to increased ROS production and subsequent cell autophagy. Therefore, it may be possible to reduce PKC-ε activation by inhibiting RACK1 expression, thereby decreasing ROS production and alleviating cell autophagy and cellular damage.

In diabetes, the activation of PKC is mediated by the formation of diacylglycerol (DAG), a physiological activator of PKC [26]. Blocking DAG, the activator of PKC, can interrupt the metabolic signaling cascade and inhibit the production of ROS. Therefore, inhibiting DAG formation is a potential method to control this signaling pathway [27]. During the onset and progression of diabetes, the inhibition of phospholipase D and phospholipase C can lead to an increase in the level of DAG through de novo synthesis. This increase in DAG levels can contribute to the induction of more severe oxidative stress in diabetes[28-30]. The enzymes phosphate hydrolase 1 and hydrolase 2 can catalyze the conversion of phosphatidic acid into DAG through a process called “de novo” synthesis [31,32]. However, because of its biochemical complexity and multiple sources, direct DAG inhibition is not the best treatment option for diabetes. RACK1, as a scaffold protein involved in multiple signal transduction cascades, can promote the expression of PKC

and enhance its activity in cells, in a manner highly dependent on PKC-ε [33,34]. This makes it a promising therapeutic target to replace DAG inhibition. By inhibiting RACK1, the expression and activity of PKC-ε can be reduced, leading to a decrease in ROS production, potentially mitigating oxidative stress in diabetes. Furthermore, high glucose and hypoxia can induce the expression of the apoptosis-promoting transcription factor C/EBP Homologous Protein in ARPE-19 cells, and disrupt the integrity of tight junctions [35]. In our study, we observed that silencing RACK1 reduced FITC leakage in ARPE-19 cells under high glucose and hypoxia conditions. However, the specific downstream mechanisms related to the changes in tight junction proteins are not yet fully understood and require further research to elucidate the underlying mechanisms by which RACK1 inhibits the protection of the outer blood-retinal barrier (oBRB).

However, this study has limitations that should be discussed. One of the main limitations of this study is that all the mechanistic experiments were conducted in ARPE-19 cells. Future studies need to confirm the effect of RACK1 on the oBRB in diabetic rats. Studies conducted solely in cell lines, such as ARPE-19 cells, have limitations and may not fully reflect the *in vivo* effects of RACK1 on the oBRB in diabetic rats or other animal models. Animal models are therefore essential for studying complex physiological processes and evaluating potential therapeutic interventions, as they provide a more comprehensive understanding of the *in vivo* effects, including the systemic factors and interactions between different cell types within the tissues of interest. Therefore, future studies should aim to confirm the effect of RACK1 on the oBRB in animal models of diabetes, such as diabetic rats. These studies may involve assessing the expression and localization of RACK1, PKC isoforms, and DAG signaling components in the retinal tissue of diabetic animals. Additionally, functional assays can be performed to evaluate the integrity and permeability of the oBRB.

CONCLUSION

Knockdown of RACK1 can reduce the activity of PKC-ε and the production of ROS, thereby alleviating cellular oxidative stress and inflammatory responses. By reducing the excessive activation of PKC-ε/ROS, the occurrence and progression of early diabetic retinopathy can be reduced. This may be achieved through the reduction of cellular oxidative stress and inflammatory response, improvement of retinal cell survival and function, and the reduction of vascular lesions and inflammatory infiltration. Therefore, inhibiting RACK1 may be a potential therapeutic strategy to slow down the progression of early diabetic retinopathy by regulating PKC-ε/ROS. However, further research is needed to determine the safety and efficacy of this strategy, and to definitively explore its potential clinical applications.

ARTICLE HIGHLIGHTS

Research background

Diabetic retinopathy (DR) is a major ocular complication of diabetes mellitus, leading to visual impairment. Retinal pigment epithelium (RPE) injury is a key component of the outer blood retinal barrier, and its damage is an important indicator of DR.

Research motivation

Therefore, inhibiting RACK1 may be a potential therapeutic strategy to slow down the progression of early diabetic retinopathy by regulating PKC-ε/ROS.

Research objectives

Knockdown of RACK1 can reduce the activity of PKC-ε and the production of ROS, thereby alleviating cellular oxidative stress and inflammatory responses. By reducing the excessive activation of PKC-ε/ROS, the occurrence and progression of early diabetic retinopathy can be reduced.

Research methods

In this study, Sprague-Dawley rats and ARPE-19 cells were used as *in vivo* and *in vitro* models, respectively, to explore the role of RACK1 in mediating PKC-ε in early DR. Furthermore, the effect on the apoptosis and barrier function of retinal pigment epithelium (RPE) cells was also investigated in the former model.

Research results

Knockdown of RACK1 can reduce the activity of PKC-ε and the production of ROS, thereby alleviating cellular oxidative stress and inflammatory responses. By reducing the excessive activation of PKC-ε/ROS, the occurrence and progression of early diabetic retinopathy can be reduced.

Research conclusions

this study proposes that by reducing the excessive activation of PKC-ε/ROS, the occurrence and progression of early diabetic retinopathy can be reduced. This may be achieved through the reduction of cellular oxidative stress and inflammatory response, improvement of retinal cell survival and function, and the reduction of vascular lesions and inflammatory infiltration.

Research perspectives

One of the main limitations of this study is that all the mechanistic experiments were conducted in ARPE-19 cells. Future studies need to confirm the effect of RACK1 on the oBRB in diabetic rats.

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